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ORIGINAL ARTICLE

Structure characterization and quantification of a new isoflavone from the arial parts of *Phyllanthus niruri*

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Abstract A new isoflavone together with 5,7-dimethoxy-3,4′-dihydroxy-3′,8-di-C-prenylflavanonol and 5,3′-dihydroxy-6,7,4′-trimethoxyflavone has been isolated from the arial parts of *Phylanthus niruri*. Based on spectral methods, the structure of the new compound was elucidated as 6-hydroxy-7,8,2′,3′,4′-pentamethoxyisoflavone. The isolated isoflavone was quantified by gas chromatography couple with mass spectroscopy (GC–MS) in selected ion monitoring (SIM) mode. Suitable methods for quantification and extraction have been developed. The quantifying limit of this method was less than 5 ng/ml in 10 g (fresh weight) of sample. Recovery of isoflavone in spiked samples exceeded 62–72% while R.S.D. ranged from 1.0% to 6.1%. The results showed significant variation in the amount of this marker in methanol extract of *Phylanthus niruri* from Kushtia, Bangladesh and Java Island, Indonesia, even though the values were almost identical for most of the cases. Isoflavone was detected in all the samples ranging from 0.12% to 0.29%.

1. Introduction

Phylanthus niruri Linn. (Euphorbiaceae), a small plant which grows mainly in tropical and subtropical regions in Central and South American countries, India, and East Asia, is one of the most important medicinal plants used by people in these countries for treatment of jaundice, asthma, hepatitis, urolithic disease, fever, malaria, stomachache, and tuberculosis (Unander et al., 1991). Extensive chemical examinations of this plant have been carried out and several constituents were isolated such as lignans, alkaloids, flavonoids, tannins, phthalic acid, gallic acid and terpenoids (Balawant et al., 1986; Joao et al., 1998).

Many antioxidant compounds from plant sources have been identified as free radical or active oxygen scavengers. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity (Miller et al., 1995). Natural antioxidants can protect the

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human body from free radicals and the progress of many chronic diseases as well as retard lipid oxidative rancidity in foods (Yildirim et al., 2001; Kehrer, 1989; Oktay et al., 2003; Tanizawa et al., 1992; Aruoma, 1994).

Reactive oxygen species (ROS) including free radicals such as superoxide anion radicals ($O^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen ($^1O_2$) is various forms of activated oxygen and often generated by oxidation product of biological reactions or exogenous factor (Halliwell et al., 1992; El-habit et al., 1990; Ito et al., 1983).

ROS have aroused significant interest among scientists in the past decade. Their broad range of effects in biological and medicinal systems has drawn on the attention of many experimental works. ROS can cause lipid peroxidation in foods, which leads to deterioration of the food (Pryor, 1991).

Intensive phytochemical examinations of this plant have been carried out. Constituents such as alkaloids, flavonoids, lignans, tannins, phenols and terpenes have been identified. However, the composition of the methanol extract, as used for medicinal purposes, has not been adequately studied. Although the specific compounds have not been precisely defined, some research results credit the therapeutic action on urinary tract stones to the phenols (Calixto et al., 1998; Ishmaru et al., 1992). Valid quality control methods need to be developed in order to comply with regulatory requirements if this plant is to be used by the pharmaceutical industry (BRAZIL, Ministry of Health, Health Surveillance Agency, 2000; EMEA, European Agency for Evaluation of Medicinal Products, 1999).

Several high performance liquid chromatographic (HPLC) methods have been reported to quantify phenolic compounds in complex biological matrices such as herbal raw materials, extracts and food products (Escarpa and Gonzalez, 2000; Keinänen and Julkunen-Titto, 1998; Wang and Helliwell, 2001). However, currently there is no method with adequate resolution to quantify substances present in the extractives of *P. niruri*. Therefore the aim of this work was to isolate and structure elucidation of new isoflavone and to validate a GC–MS method applied to the quality control of *P. niruri* both as raw material and as a technological intermediate product.

2. Experimental

2.1. General

IR spectra were recorded (KBr disks) on a FT-IR spectrophotometer, validation (ν_{max} in cm^{-1}). ^1H NMR spectra were recorded on a Bruker R-32 (300 MHz) instrument in $CDCl_3$ and DMSO-d_6 with TMS as an internal standard (chemical shifts in δ, ppm). UV spectra were recorded on HATACHI, U-2000 spectrophotometer Ultrospeck in methanol (λ_{max} in nm). The entire chemical is analytical reagent grade. TLC was performed using silica gel GF_{254}. Anhydrous sodium sulfate (Merck, Germany) was cleaned by heating at 200 °C before use. Silica gel (60–120 mesh, Loba, India) was activated at 400 °C for 12 h. prior to use. Stock solutions of each analyte (1000 μg/ml) were prepared in methanol. Mixtures of the analytes for working standard preparation and sample fortification were also prepared in methanol. All stock solutions and mixtures were stored at −10 °C in the dark.

2.2. Plant material

The arial parts of *P. niruri* were collected from the Kushtia district and also collected from Jawa Island, Indonesia. Both the plants were identified and voucher specimen (BD 32) was deposited in the herbarium of the School of Biology, University Sains Malaysia. The plant was dried at 40 °C for a week in an air oven. The separated leaves and branches were reduced in a knife mill.

2.3. Extraction and isolation

Dried arial parts of the plant (1 kg) were milled into powder and then extracted with methanol (8 L) in a Soxhlet extractor for 36 h. The extract was evaporated in a rotatory evaporator and dried by vacuum pump. The methanolic extract (40 g) was suspended on water and extracted successively with hexane, chloroform, ethyl acetate, and butanol to yield hexane (3 g), chloroform (11.2 g), ethyl acetate (6.8 g) and BuOH-soluble (4.88 g) fractions, respectively. The chloroform soluble fraction (5 g) was subjected to chromatography on silica gel (60–120 mesh, Merck) and was eluted with ethyl acetate–hexane–acetone (4:3:1) solvent system to give one major colorless amorphous powder (7.8 mg) of compound **1** and two minor compounds of 5,7-dimethoxy-3,4′-dihydroxy-3′,8-di-C-prenyl-flavanonol and 5,3′-dihydroxy-6,7,4′-trimethoxyflavone (Hossain et al., 2009).

2.4. Compound (Fig. 1)

The purified compound was crystallized from hexane–methanol to give colorless amorphous powder (7.8 mg), R_f 0.59 (hexane–chloroform–acetone; 85:12:3); (M^+, 387), UV (MeOH): λ_{max} = 255, 335 nm; IR ν_{max} = 3415, 3250, 2910, 2840, 2343, 2131, 1645, 1550, 1470, 1365, 1100, 1050, 1020, 984, 928 cm^{-1} ; ^1H NMR: δ 8.05 (s, 1H, H-2), 7.22 (s, 1H, H-5), 6.95 (s, 1H, H-6′), 6.85 (s, 1H, H-5′), 3.90 (s, 3H, 8-OCH_3), 3.94 (s, 3H, 7-OCH_3), 3.89 (s, 3H, 2′-OCH_3), 3.95 (s, 3H, 3′-OCH_3), 3.97 (s, 3H, 4′-OCH_3), 12.75 (s, 1H, –OH). ^{13}C NMR: δ 182.5 (C-4), 165.6 (C-7), 164.5 (C-5), 159.7 (C-8), 155.9 (C-2), 154.0 (C-2′), 152.4 (C-4′), 144.5 (C-5′), 124.9 (C-3), 118.2 (C-6′), 112.7 (C-1′), 100.0 (C-6 and C-3′), 95.7 (C-8), 57.8 (OCH_3), 57.6 (OCH_3), 57.4 (OCH_3), 57.3 (OCH_3), 57.1 (OCH_3). Found C = 62.01, H = 4.90%; $C_{20}H_{19}O_8$ requires, C = 62.17, H = 4.78%.

2.5. GC–MS analysis

2.5.1. Preparation of samples from market for GC–MS analyses

The methanol, hexane, chloroform and ethyl acetate extract (1 ml) were diluted with 5 ml of methanol and the samples were filtered through 0.45 lm membrane filters (Molsheim, France) prior to GC–MS analysis.

2.5.2. Identification and quantification of marker in the samples by GC–MS

The GC–MS analysis of the methanol, hexane, chloroform and ethyl acetate extract of *P. niruri* was performed using a Varian GC–MS (Model Varian CP 3800, USA) equipped with a VF-5 fused silica capillary column (30 m × 0.25 i.d. mm lm

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thickness 0.25 μm, Varian, USA). For GC–MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as a carrier gas at a constant flow rate of 1 ml/min. Injector and mass transfer line temperature were set at 250 and 300 °C, respectively. The oven temperature was programmed from 50 to 200 at 8 °C/min, and then held isothermal for 20 min and finally raised to 300 °C at 10 °C/min. Diluted samples (1/100 v/v, in methanol) of 0.2 μl were manually injected in the split less mode. Identification of compounds of the crude extracts was based on GC retention time on VF-5 capillary column, computer matching of mass spectra with standards (Mainlab, Replib and Tutorial data of GC–MS systems). The reference compound, isoflavone was used as marker. The marker was accurately weighed and dissolved in methanol to produce a series of concentrations. Standard calibration curves were established by plotting the peak areas against different concentrations of the reference compound (varying from 5.0 to 1000 ng on column for isoflavone. The external standard method was used for quantification of the marker in the different types of crude extracts of *P. niruri* samples from the district of Kushtia in Bangladesh.

The system suitability of the method was evaluated by the intra- and inter-day precision and accuracy of replicates. The accuracy was evaluated through recovery studies by adding known amounts of the standard solution to the extract. Controls from all samples were prepared and analyzed. The recovery experiment was performed at three different standard concentrations.

3. Results and discussion

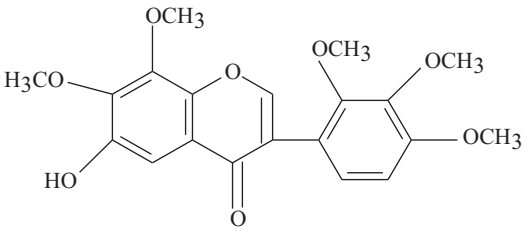
3.1. Sample collection

The *P. niruri* samples were collected during early morning, when the plants were fresh. After collection, the sample was kept in a polyethylene bag with aluminum foil protected cover and stored in refrigerator 4 °C at to avoid any deterioration.

3.2. Extraction

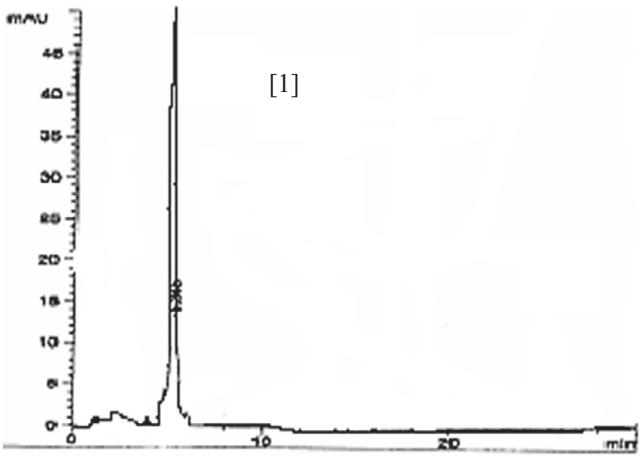
The dried plant samples were pulverized into powder form. The dried arial parts of *P. niruri* were extracted with methanol in a Soxhlet extractor. The crude methanol extract was suspended in water and extracted successively with hexane, chloroform, ethyl acetate, and butanol. The layers were collected and dried by $NaSO_4$. It was then filtered and the filtrate was evaporated near to dryness by modified Kuderna–Danish evaporator for GC–MS analyses. The chloroform extract was subjected to column chromatography on silica gel and eluted with a hexane–acetone mixture. By repeated chromatography and preparative TLC, two compounds were isolated from one fraction of the chloroform extract. 6-Hydroxy-7,8,2′,3′,4′-pentamethoxyisoflavone (Fig. 1) was isolated from this plant for the first time. To our knowledge Fig. 1 has not been reported previously from any plant source.

The compound (Fig. 1) was obtained as a yellowish powder. It had the molecular ion peak at $[M]^+$ *m/z* 387 and the elemental analysis showed C 62.01%, H 4.90%, which corresponds to the molecular formula $C_{20}H_{19}O_8$. The UV spectrum of Fig. 1 exhibited absorptions at 255 nm and 335 (sh) nm, indicative of its isoflavone nature (Markhman, 1992). The IR spectrum of compound (Fig. 1) showed the presence of a hydroxyl group at 3415 cm^{-1}, ketonic group at 1645 cm^{-1} and other bands at 1550 and 1470 cm^{-1}, assignable to an aromatic ring. This was supported by ^1H NMR and ^{13}C NMR spectra, which showed resonances, characteristic of isoflavone skeleton (Agarwal, 1998; Mabry et al., 1970) at δ 8.05 for H-2 and at 155.9, 124.9 and 182.5 ppm, respectively for 2, 3 and 4 carbons. The ^1H NMR spectrum of the compound Fig. 1 showed three proton singlets at δ 7.22, 6.95 and 6.85 assigned to H-5, H-6′ and H-5′ protons, respectively, confirming that Fig. 1 is a hexasubstituted isoflavone. Additionally, the ^1H NMR showed the resonance signals of five methoxy singlets at δ 3.97, 3.95, 3.94, 3.90 and 3.89. The fragments at *m/z* 167 and 220 formed after retro-Diel Alder cleavage, indicated that Fig. 1 has two methoxy and hydroxy groups in ring A and three methoxy groups in ring B. The presence of M-31 peak (Campbell et al., 1969; Dewick et al., 1972) in Fig. 1 in high abundance in the MS indicated 2′-methxyl in Fig. 1. The six protons at 3.89 and 3.90, indicated an aromatic methoxyl groups, were assigned to the 7 and 8 positions and confirmed by the fact that there was no bathochromic shift in the UV absorption maximum with NaOAc. Since compound Fig. 1 did not show bathochromic shift on treatment with $AlCl_3$, it was inferred that it contained free –OH at C-6 rather than at C-5. The three methoxyls were assigned to C-2′, C-3′ and C-4′ carbons of B ring and confirmed by the carbon resonances at 154.0 (C-2′), 100.0 (C-3′) and 144.5 (C-5′) ppm, which were in consistent with such oxygenation pattern (Nkengfack et al., 1989). The broad band decoupled ^{13}C NMR spectrum showed



1

Figure 1 Structure of marker.



[1]

Figure 2 Chromatogram of reference compounds, isoflavone.

Table 1 Analytical characteristics of calibration curves for the marker isoflavone.

Standard	Ret. time (min)	Range (ng/ml)	Regression equation	Detection limit (ng)	R^2	Precision (%) (SD[a]) (n = 5, 10 ng/μl)	
						Inter-day	Intra-day
Isoflavone	6.37	5–1000	$0.1919x - 0.1253$	2.5	0.9978	0.71	0.31

[a] SD = Standard deviation.

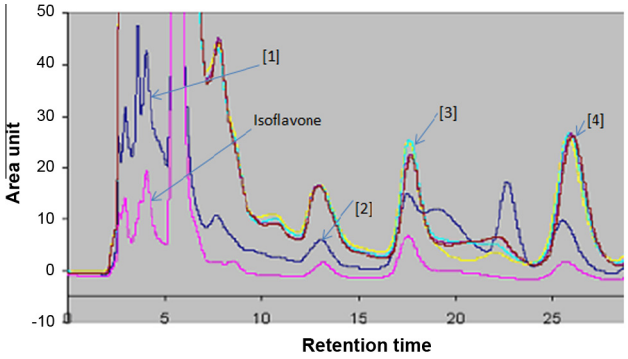


Figure 3 Isoflavone; 1, Methanol extract; 2, Hexane extract; 3, Ethyl acetate extract; 4, Chloroform extract.

Table 2 Recovery of isoflavone from spiked samples.

Analyte	Recovery (%) Isoflavone
Methanol extract	62[a]
Hexane extract	64
Ethyl acetate extract	71
Chloroform extract	73
Blank	70[a]

[a] The average spiked recovery.

Table 3 Percent concentrations of marker isoflavone in different extract samples from *Phyllanthus niruri*.

Name of extracts	Marker concentration (% of total dry weight)
	Isoflavone
Methanol extract	0.29 ± 0.08
Hexane extract	0.12 ± 0.07
Ethyl acetate extract	0.24 ± 0.09
Chloroform extract	0.25 ± 0.10

20 carbon signals. The analysis of this spectrum by the aid of DEPT technique unequivocally indicated that Fig. 1 contains ten quaternary carbons, which include one carbonyl carbon (182.5 ppm), four CH carbons and five CH_3 carbons. Based on the above spectral data, the structure of Fig. 1 was established as 6-hydroxy-7,8,2′,3′,4′-pentamethoxyisoflavone.

3.3. Concentrations of the marker in extracts of P. niruri samples

The GC–MS method applied is a modification of that reported by Stuart and Sakmann (1994) for the analysis of isoflavone present in the crude extract samples. In the present study, a programmed method was used for simultaneous assay of the authentic markers for which chemical structures are shown in Fig. 1. All standards were determined in a single GC–MS run. The standards were resolved and eluted at 6.37 min. (Fig. 2). The marker (5, 50, 500, 750 and 1000 ng on column for isoflavone) showed a good linearity in the range from 5.0 to 1000 ng in the calibration curves that were obtained by GC–MS analysis (Table 1). The reference marker was present in the chromatographic profiles of the samples from various extracts when the sample solution was analyzed by GC–MS (Fig. 3). The peak of isoflavone was confirmed by comparison of their retention times with reference standard.

To assess the precision of these methods, standard solutions of isoflavone were determined six times on the same day and over a six-day period. The results showed a very good precision, ranging from 5 to 1000 μg/ml (Table 1). The accuracy of the method was evaluated through recovery studies. The recovery experiments were performed at three concentrations (5, 50 and 100 ng) of the standard added to sample solutions, in which the marker content had been determined, using a sample from the district of Kushtia in Bangladesh.

The results for the recoveries of isoflavone were in the range of 62–73% (Table 2). The limit of detection (LOD) of the GC–MS method, established at signals three times that of the noise for isoflavone was 2.5 ng, respectively. The method validation was carried out via recovery study by using three replicate 10 g subsamples spiked to yield the final concentrations of 5, 50 and 100 ng/g for isoflavone. The precision of the method, as indicated by the relative standard deviation (R.S.D) of the recovery, was assessed using three independent pretreatment and extractions of analytes from samples. Average recovery ranged from 62% to 73% with good R.S.D. ranging from 1% to 10%, indicating good recovery and repeatability of the method (Table 2). Fig. 3 displays typical SIM chromatograms of isoflavone detected in almost all the crude extract samples. The peaks were identified and quantitated using retention times with their characteristic ions of SIM and response factors, respectively. Positively identified target compound in every extract were analyzed three replicate.

The GC–MS procedure was applied to the determination of the marker in the different crude extracts. As shown in Table 3, all the analyzed samples showed a significant range in the concentrations of the marker, in different crude extract samples from the same place. The variation may be ascribed to environmental conditions and variation in sample sourcing.

Concentrations of isoflavone ranged from 0.12% to 0.29% (Table 3). However, the level of the marker concentration of isoflavone was considerably higher in methanol (0.29%) followed by chloroform (0.25%), ethyl acetate (0.24%) and hexane extract (0.12%).

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4. Conclusions

The GC–MS chromatographic profiles of the extracts of *P. niruri* collected from the district of Kushtia were qualitatively almost similar but the results showed variations in the concentrations of the marker, isoflavone. The GC–MS finger-printing could be used in authentication of extract samples and formulations.

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References

Agarwal, P.K., 1998. Carbon-13 NMR of Flavonoids. Elsevier Science, Amsterdam.
Aruoma, O.I., 1994. Food Chem. Toxicol. 62, 671–683.
Balawant, S.J., William, S.P., Goppinath, K., Krishna, B., 1986. J. Nat. Prod. 32, 234–241.
BRAZIL, Ministry of Health, Health Surveillance Agency, 2000. Resolution NMR. 2000, Diário Oficial da Unia~o, 24 Abr 2000.
Calixto, J.B., Santos, A.R.S., Cechinel-Filho, V., Yunes, R.A., 1998. Med. Res. Rev. 18, 225–258.
Campbell, R.V.M., Harper, S.M., Kemp, A.D., 1969. J. Chem. Soc., 1787–1791
Dewick, P.M., Harborne, J.B., Mabry, T.J., 1972. The Flavonoids, Advances in Research. Chapman and Hall, London.
El-habit, O.H.M., Saad, H.N., Azab, K.S., 1990. Mut. Res. 466, 179–186.
EMEA, European Agency for Evaluation of Medicinal Products, 1999. Note for Guidance on Specifications: Test Procedures and Acceptance Criteria for Herbal Drugs. Herbal Drug Preparations and Herbal Medicinal Products, London (EMEA/HMPWP/1999).
Escarpa, A., Gonzalez, M.C., 2000. J. Chromatogr. A 897, 161–170.
Halliwell, B., Gutteridge, J.M.C., Cross, C.E., 1992. J. Lab. Clin. Med. 119, 598–620.
Hossain, M.A., Ahameda, A., Ismail, Z., 2009. Asian J. Food Agric. Ind. 9 (1), 345–356.
Ishmaru, K., Yoshimatsu, K., Yamakawa, T., Kamada, H., Shimomura, K., 1992. Phytochemistry 31, 2015–2018.
Ito, N., Fukushima, S., Hasegawa, A., Shibata, M., Ogiso, T., 1983. J. Natl. Can. Inst. 70, 343–347.
Joao, B., Calixto, A.R., Santos, S., Filbo, V.C., Rosendo, A.Y., 1998. Med. Res. Rev. 18 (4), 225–231.
Kehrer, J.P., 1989. Rev. Toxicol. 23, 21–48.
Keinänen, M., Julkunen-Titto, R., 1998. J. Chromatogr. A 793, 370–377.
Mabry, T.J., Markham, K.R., Thomas, M.B., 1970. The Systematic Identification of Falvonoids. Springer, Berlin.
Markhman, K.R., 1992. In Techniques of Flavonoid Identification. Academic Press, New York, pp. 39–82.
Miller, N.J., Diplock, A.T., Rice-Evans, C.A., 1995. J. Agric. Food Chem. 43, 1794–1801.
Nkengfack, A.E., Samson, D.R., Tempesta, M.S., Fomum, Z.T., 1989. J. Nat. Prod. 52, 320–324.
Oktay, M., Gulcin, I., Küfrevioglu, Ö.I., 2003. Lebensm-Wiss. U-Technol. 36, 263–271.
Pryor, W.A., 1991. Am. J. Clin. Nut. 53, 391–393.
Stuart, G.J., Sakmann, B., 1994. Nature 367, 69–72.
Tanizawa, H., Ohkawa, Y., Takino, Y., Ueno, A., Kageyama, T., Hara, S., 1992. Chem. Pharmacol. Bull. 40, 1940–1944.
Unander, D.W., Webster, G.L., Blumberg, B.S., 1991. J. Ethnopharmacol. 34, 97–133.
Wang, H., Helliwell, K., 2001. Food Res. Int. 34, 223–227.
Yildirim, A., Mavi, A., Kara, A.A., 2001. J. Agric. Food Chem. 49, 4083–4089.

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